Tamoxifen disrupts the reproductive process in gilthead seabream males and modulates the effects promoted by 17α-ethynylestradiol

Running Title: Effect of Tmx alone or combined with EE_2 on hermaphrodite fish

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Abstract

17α-ethynylestradiol (EE_2), which is used in oral contraceptives and hormone replacement therapy, is a well documented estrogenic endocrine disruptor and an aquatic contaminant. In the present study, adult male specimens of the marine hermaphrodite teleost gilthead (*Sparus aurata* L.) were fed a diet containing tamoxifen (Tmx), an estrogen receptor ligand used in cancer therapy, alone or combined with EE_2, for 25 days and then fed a commercial diet for a further 25 days (recovery period). The effects of short (5 days) and long (25 days) treatments on several reproductive and gonad immune parameters and the reversibility of the disruptive effects after the recovery period were examined. Our data showed that Tmx acted as an estrogenic endocrine disruptor as revealed by the increase in the hepatic transcription of the *vitellogenin* gene in males, the serum levels of 17β-estradiol and the gonad expression levels of the estrogen receptor α and G protein-coupled estrogen receptor genes, and the recruitment of leukocytes into the gonad, a well known estrogenic-dependent process in gilthead seabream males. On the other hand, Tmx also increased sperm concentration and motility as well as the serum levels of androgens and the expression levels of genes that codify for androgenic enzymes, while decreasing the expression levels of the gene that code for gonadal aromatase. When applied simultaneously, Tmx and EE_2 could act in synergy or counteract, each other, depending on the parameter measured. The disruptive effect of EE_2 and/or Tmx was not reversible after a 25 day recovery period.

Keywords: Tamoxifen, 17α-ethynylestradiol, endocrine disruption reversibility, spermatogenesis, steroidogenesis, gilthead seabream.

Summary statement

In gilthead seabream males, Tmx disrupts the reproductive process including the gonad immune response and counteracts or enhances the effects of EE_2. A 25-day recovery period did not reverse these effects in adult males.

Introduction

Endocrine disrupting chemicals (EDCs) exert their effects via agonistic/antagonistic interactions with hormone receptors or by interfering with the normal synthesis, transport, metabolism, and secretion of endogenous hormones (Segner et al., 2006). Among EDCs, the most studied are the compounds that interfere with estrogen receptors (ERs), which have hazardous and estrogenic effects on fish reproduction (Folmar et al., 1996; Jobling et al., 1998, 2002; Hassanin et al., 2002; Penáz et al., 2005). Some of these compounds are pharmaceutical products released in waste waters which reach the aquatic environment through sewage treatment effluents (Mills and Chichester, 2005).

In the group of estrogenic EDCs, 17α-ethynylestradiol (EE_2), a major constituent of contraceptive pills (Owen and Jobling, 2012), has a higher binding affinity to ERs than natural 17β-estradiol (E_2) (Blair et al., 2000) and is one of the most potent compounds in the aquatic environment. Low concentrations of EE_2 (3-17 ng/L) are sufficient to induce vitellogenin (Vtg) production in male fish (Holbech et al., 2001; Rose et al., 2002; Andersen et al., 2003), to modify sexual behaviour (Coe et al., 2010; Reyhanian et al., 2011; Filby et al., 2012) and to disrupt the reproductive capacities of fish (Nash et al., 2004; Pawlowski et al., 2004; Fenske et al., 2005; Schäfers et al., 2007). However, the ability of fish to recover from estrogen exposure has drawn little attention, apart from some studies on sexual differentiation and reproductive capacity (Hill and Janz, 2003; Nash et al., 2004; Schäfers et al., 2007; Larsen et al., 2009; Baumann et al., 2014).

Other EDCs is tamoxifen (Tmx) which is widely used as a drug in cancer therapy. Studies in humans have shown that approximately 65% of administered Tmx is excreted with faeces, while its active metabolite OH-Tmx is excreted with bile and urine. Tmx is a nuclear ER ligand which in mammals, acts as estrogen agonist on some cell types but as an antagonist or partial agonist on others, which reflects the diversity of the mechanisms that mediate ER actions in different tissues (Fitts et al., 2011). In addition, Tmx acts as an agonist on the G protein-coupled estrogen receptor (GPER), a transmembrane receptor that mediates rapid responses of estrogen and is widely expressed in estrogen target tissues (Revankar et al., 2005), including fish testis and ovary (Liu et al., 2009; Pang and Thomas, 2010). However, the effects of Tmx and its action mechanisms in fish are just beginning to be understood, in part because of the interest that binary mixtures of EDCs has attracted in the recent years (Sun et al., 2009,

2011a, 2011,b). Such studies have been performed in gonochoristic fish and showed that the estrogenic or anti-estrogenic effects of Tmx depend on the gender, concentration and tissue analyzed (Leaños-Castañeda et al., 2002; Chikae et al., 2004; Sun et al., 2011a, 2011b). Tmx treatment leads to the masculinization of genetic female fish (Kitano et al., 2007; Hulak et al., 2010; Liu et al., 2010). Moreover and although the effects of Tmx mask or neutralize many signs of estrogen exposure, the impairment of the fish reproductive process is not restored (Santos et al., 2006; Elias et al., 2007; van der Ven et al., 2007; Sun et al., 2009). To the best of our knowledge, no such studies have been studied in hermaphrodite fish such as gilthead seabream.

The gilthead seabream (*Sparus aurata* L.) is a marine, seasonally breeding, protandrous teleost that develop a functional testicular area near by an immature previtellogenic ovary during the first two reproductive cycles. We have recently reported that EE_2 dietary intake increases the hepatic expression levels of *vtg,* disrupts spermatogenesis and promotes leukocyte infiltration in the gonad (Cabas et al., 2011, 2013), a physiological process needed for gonad renewal after spawning (Chaves-Pozo et al, 2005a, 2005b; Liarte et al., 2007). Moreover, most of these effects vary with the reproductive stage of the specimens (Cabas et al., 2011, 2013). On the other hand, the dietary intake of Tmx has been shown to be a suitable approach for studying its potentially endocrine disruptive effects (Benninghoff and Williams, 2008; Singh et al., 2014).

In the present study, we investigate the effect of the dietary intake of Tmx alone or in combination with EE_2 on some reproductive events in gilthead seabream and the possible reversibility of these effects after a recovery period of 25 days, during which fish were again fed with a commercial diet. This approach, as a way to unbalance the endocrine status of the fish, would improve our understanding of the complex network acting on the regulation of the reproductive function in this species, which has a great commercial interest in the Mediterranean area.

Material and Methods

Healthy specimens of gilthead seabream (Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain).

The experiment was performed using 80 male specimens of gilthead seabream, all in the spermatogenesis stage, with a mean body weight of 215 ± 6.5 g. Fish were

kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 14.6 to 17.8°C. Environmental parameters, mortality and food intake were recorded daily. The EE_2 (98% purity; Sigma) and Tmx (Sigma) were incorporated in the commercial feed (44% protein, 22% lipids, Skretting, Spain) at doses of 0 (control), 5 μg EE_2/g food, 100 μg Tmx/g food or 5 μg EE_2 + 100 μg Tmx/g food, using the ethanol evaporation method (0.3 L ethanol/kg of food) as described elsewhere (Shved et al., 2007). The concentration of EE_2 used in this study was previously assayed and shown to be the lowest concentrations producing an effect on some reproductive events of gilthead seabream (Cabas et al., 2011, 2013), while the concentration of Tmx used was twenty-fold greater than the concentration of EE_2 in order to guarantee a Tmx-ER interaction, considering that Tmx has a lower affinity than EE_2 to bind ER (Denny et al., 2005). In any case, the Tmx concentration used in this study is similar to, or lower than, those tested in previous investigations (Chikae et al., 2004; Hulak et al., 2010).

The specimens were fed with EE_2 and/or Tmx supplemented feed for 25 day, after which they were fed with the commercial food for a further 25 days (recovery period). The specimens were fed *ad libitum* three times a day and fasted for 24 h before sampling, which was carried out after 5 and 25 days of the EE_2 and/or Tmx exposure and after the recovery period (n=6 fish/group and time). Specimens were anesthetized with 40 μL/L of clove oil and the urogenital pore was dried before collecting sperm as described below. The specimens were then weighed, decapitated, and the gonads removed and weighed. Fragments of liver and gonad were processed for gene analysis and light microscopy, as described below. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80°C until use.

The experiments comply with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committee of the University of Murcia (Spain) and that of the "Instituto Español de Oceanografía" (Spain) for the use of laboratory animals.

Analysis of gene expression

Total RNA was extracted from liver and gonad fragments with TRIzol Reagent (Invitrogen, Barcelona, Spain) following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA of five fish per group was independently treated with DNase I (amplification grade, 1 unit/μg RNA, Invitrogen, Barcelona, Spain) to remove genomic DNA traces that might interfere with the PCR

reactions, and the SuperScript III RNase H−Reverse Transcriptase (Invitrogen, Barcelona, Spain) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 μg of total RNA, at 50°C for 50 min.

Real-time PCR was performed with an ABI PRISM 7500 (Applied Biosystems, Madrid, Spain) using SYBR Green PCR Core Reagents (Applied Biosystems, Madrid, Spain) and used to analyze the expression of the genes coding for (i) hepatic vitellogenin (*vtg*); (ii) steroidogenesis-related molecules: steroidogenic acute regulatory protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), steroid 11-beta-hydroxylase (*cyp11b1*), 11β-hydroxysteroid deshydrogenase (*hsd11b*), aromatase (*cyp19a1a*), 5α reductase (*srd5a*) and 3β-hydroxysteroid deshydrogenase (*hsd3b*); (iii) testicular specific protein, double sex-and mab3-related transcription factor 1 (*dmrt1*); (iv) hormone receptors: follicle stimulating hormone (FSH) receptor (*fshr*), luteinizing hormone (LH) receptor (*lhr*) and estrogen receptor α (*era*), G protein-coupled estrogen receptor (*gper*); (v) immune-relevant molecules: interleukin 1β (*il1b*), tumor necrosis factor α (*tnfa*), transforming growth factor β1 (*tgfb1*), matrix metalloproteinase (*mmp*) 9 and 13 (*mmp13*), major histocompatibility complex I α protein (*mhc1a*) and toll-like receptor 9 (*tlr9*). For each mRNA, gene expression was normalized to the ribosomal protein S18 gene (*rsp18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (where Ct is a cycle threshold). The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed in triplicate.

Analytical techniques

Serum levels of testosterone (T), 11-ketotestosterone (11KT) and E_2 were quantified by ELISA following the method described by Rodríguez et al. (2000) and previously used in gilthead seabream (Chaves-Pozo et al., 2008). Steroids were extracted from 20 μL of serum in 0.6 mL of methanol (Panreac). The methanol was then evaporated at 37°C and the steroids were resuspended in 400 μL of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1% albumin from bovine serum (Sigma)]. By using 50 μL in each well, 2.5 μL of serum was used in each well for all the assays. The T, 11KT and E_2 standards, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13×10^{-4} to 2.5 ng/mL (0.03-125 pg/well) was established in all the assays. Standards and extracted serum samples were run in duplicate. The lower limit of detection for all the

assays was 12.21 pg/mL. The intra-assay coefficients of variation (calculated from duplicate samples) were 8.26% ± 1.33% for T, 8.80% ± 1.68% for 11KT and 3.98% ± 0.57% for E_2. Details on cross-reactivity for specific antibodies were provided by the supplier (2.2% of anti-T reacts with 11KT; 0.01% of anti-11KT reacts with T; 0.1% of anti-E_2 reacts with T).

Measurement of the sperm volume, concentration and motility

Stripped sperm was obtained by gentle abdominal massage, the sperm being collected and measured in the genital pore with a syringe as the semen flowed out (urine-contaminated samples were discarded). The total semen from each fish (n=6 fish/group and time) was used immediately to determine cell concentration and motility. To determine the sperm concentration, semen was diluted in 1% formol (Panreac) and 5% $NaHCO_3$ (Sigma) in water at a ratio of 1:400 and the spermatozoa were counted using a Newbauer chamber. Motility was analyzed by activating 1 µL of sperm (diluted in Ringer 200 mOsm solution at the optimal dilution of 1:5 with 20 µL of seawater (Chereguini et al., 1997). The duration of sperm motility was determined by measuring the time elapsing between the initiation of sperm motility and the cessation of cell displacement using a light microscope at 400× magnification. The motility index was expressed on a relative scale of 0 to 5 (Sánchez-Rodríguez, 1975).

Light microscopy and immunocytochemical staining

The gonads were fixed in Bouin's solution, embedded in Paraplast Plus (Sherwood Medical, Athy, Ireland), and sectioned at 5 µm. After dewaxing and rehydratation, some sections were stained with hematoxylin-eosin in order to determine the reproductive stage and the degree of development of each specimen. Some sections were used to: i) analyze cell proliferation with a commercial mammalian antibody specific to proliferating cell nuclear antigen (PCNA, Sigma) or ii) localize acidophilic granulocytes with a monoclonal antibody (mAb) specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al., 2002) and B lymphocytes with a commercial mAb specific to immunoglobulin M (IgM, Aquatic Diagnostic) (Sepulcre et al., 2011) following an indirect immunocytochemical method previously described (Chaves-Pozo et al., 2007). Rabbit polyclonal anti-PCNA cross-reacts with PCNA from all vertebrate species investigated to date, including fish (Kilemade et al., 2002). The antibodies were used at the optimal dilutions of 1:1000, 1:100 or 1:250, respectively. No immunostaining was observed when the first antiserum was omitted.

Calculation and statistics

All data were analyzed by one-way ANOVA and a post hoc test (Tukey Honestly Significant Difference) to determine differences between groups ($P \leq 0.05$). Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. The stripped sperm volume, and the sperm concentration and motility index data were analyzed by a Student t-test to determine differences between untreated control and the treated group for each time point. The critical value for statistical significance was taken as $P \leq 0.05$. The asterisks mean: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. All statistical analyses were carried out using the GraphPad Prism 5 program.

Results

Tmx up-regulates the hepatic expression of vitellogenin gene to a lesser extent than EE_2

The hepatic expression of *vtg* gene was up-regulated after the dietary intake of EE_2 and Tmx. The Tmx-induced up-regulation was lower than that promoted by EE_2 (Fig. 1A). After the recovering period, expression levels of *vtg* remained high, although the differences that existed between treatments became less pronounced (Fig. 1A). Interestingly, Tmx decreased the levels of *vtg* transcription triggered by EE_2 when they were applied together at all time points analyzed (Fig. 1A).

EE_2 and Tmx differently affect sex steroid serum levels and the expression levels of some steroidogenic enzyme genes

After 5 days of Tmx treatment, the serum levels of T (Fig. 1B), 11KT (Fig. 1C) and E_2 (Fig. 1D) where higher than those of control ones, while EE_2 exposure had no effect in this respect (Fig. 1B-D). Interestingly, the administration of EE_2+Tmx prevented the increases in serum steroid levels induced by Tmx (Fig. 1B-D). No differences in T, 11KT or E_2 serum levels were observed after 25 days of any treatment used (Fig. 1B-D). By the end of the recovery period, T levels had fallen in the EE_2 treated fish (Fig. 1B) and E_2 serum levels had increased in Tmx treated fish (Fig. 1D); however, these effects were annulled in the fish fed with EE_2+Tmx (Fig. 1D).

Although no effect was observed in the expression levels of *star* gene after 5 days of EE_2 or Tmx dietary intake, after 25 days, they were down-regulated in the gonad of EE_2 treated fish and up-regulated in the gonad of Tmx treated fish (Fig. 2A). However, in EE_2+Tmx treated fish the transcription levels of *star* decreased at day 5

and increased at day 25 of exposure (Fig. 2A). After the recovery period, they remained similar to those of control in all treatment groups (Fig. 2A).

Regarding the transcription of the genes coding for the steroidogenic enzymes studied (Fig. 2B-G), *cyp11a1*, *hsd11b*, *srd5a* and *hsd3b* expression levels were down-regulated after 5 days of exposure to all the treatments (Fig. 2B,D,F,G). However, after 25 days, EE_2 down-regulated the expression of *cyp11b1*, *hsd11b*, *srd5a* and *hsd3b* genes (Fig. 2C,D,F,G), Tmx up-regulated the *hsd11b*, *srd5a* and *hsd3b* genes (Fig. 2D,F,G) and EE_2+Tmx up-regulated the expression of *cyp11a1*, *hsd11b*, *srd5a* and *hsd3b* genes (Fig. 2B,D,F,G). Interestingly, the expression of *cyp19a1a* was down-regulated by Tmx and up-regulated by EE_2 and EE_2+Tmx (Fig. 2E). At the end of the recovery period, the expression levels of all these genes, with the exception of the *cyp11a1,* were up-regulated in EE_2 treated fish, (Fig. 2B), while any effect depended on the gene in question in Tmx and EE_2+Tmx treated fish (Fig. 2B-G). Thus, the transcription of *cyp11a1*, *cyp11b1* and *cyp19a1a* (Fig. 2B,C,E) was down-regulated, while the transcription of *hsd11b* and *hsd3b* (Fig. 2D,G) was up-regulated, in Tmx treated fish. Expression levels of *cyp11a1* and *hsd3b* (Fig. 2B,G) were similar in Tmx and EE_2+Tmx treated fish, while those of *cyp11b1* and *srd5a* were similar in the control and EE_2+Tmx treated fish (Fig. 2C,F). On the other hand, the expression of *hsd11b* and *cyp19a1a* in the EE_2+Tmx treated fish was higher than in the control and lower than in EE_2 treated fish (Fig. 2D,E).

EE_2 reduces seminal fluid volume and sperm concentration, while Tmx increases sperm concentration and motility

After 5 days of treatment, EE_2 decreased the volume of seminal fluid to such an extent that it was not possible to measure sperm concentration or motility (Table 2). Tmx did not affect the volume of seminal fluid, or the sperm concentration or motility and EE_2+Tmx did not affect seminal fluid volume or sperm motility but decreased the sperm concentration (Table 2). After 25 days of EE_2 or Tmx treatments, seminal fluid volume was unaffected but respective decrease and increase in sperm concentration were evident. However, fish treated with EE_2+Tmx showed a decreased seminal fluid volume and sperm concentration. Sperm motility was increased only in Tmx treated fish (Table 2). Moreover, no detectable seminal fluid was observed after the recovery period in EE_2 or EE_2+Tmx treated fish, while in Tmx treated fish both the seminal fluid volume and sperm concentration were higher than in control fish (Table 2).

EE_2 and Tmx affect spermatogenesis in a different way

The control fish remained in spermatogenesis throughout the experimental period. The testicular area of the gonad was formed by tubules with a germinal epithelium consisting of spermatogonia stem cells and cysts of germ cells in all developmental stages (spermatogonia, spermatocytes and spermatids); a varying amount of free spermatozoa was also observed in the lumen of the tubules (Fig. 3A). Interestingly, scattered degenerative cysts with very condensed genetic material were observed (Fig. 3A).

Although the general morphology of the testicular area did not change after 5 days of EE_2 (Fig. 3B) or EE_2+Tmx treatment, more degenerative cysts were evident than in control fish. However, after 25 days, the germinal epithelium was made up of spermatogonia stem cells and some cysts of primary spermatogonia and no meiotic germ cells were observed (Fig. 3C). After the recovery period, the germinal epithelium of these two groups of fish still consisted of spermatogonia and Sertoli cells and abundant interstitial tissue (Fig. 3D,E). However, while most of the tubules had a collapsed lumen in EE_2 treated fish (Fig. 3D), some tubules with a small amount of free spermatozoa were observed in the EE_2+Tmx treated fish (Fig. 3E). Testis morphology in fish fed with Tmx did not show any noticeable change compared with that of control fish during the treatment period or after resuming the commercial diet (data not shown).

Cysts of proliferating spermatogonia and spermatocytes were observed in control fish (Fig. 4A) and in Tmx exposed fish (Fig. 4B) at all the time points analyzed. However, in fish treated with EE_2 (Fig. 4C) and EE_2+Tmx, only some Sertoli cells were immunolabelled with anti-PCNA after 25 days of treatment. Interestingly, after the recovery period, while a few spermatogonia and Sertoli cells were seen to divide in the testis of EE_2 treated fish (Fig. 4D), numerous cysts of proliferating spermatogonia and some proliferative Sertoli cells were observed in the testis of EE_2+Tmx treated fish (Fig. 4E).

Five days of EE_2, Tmx or EE_2+Tmx treatment promoted a decrease in *dmrt1* gene expression, the EE_2 treated group showing the strongest effect (Fig. 4F). However, after 25 days of treatment, the expression level of *dmrt1* gene remained down-regulated in EE_2-treated fish, while it was up-regulated in Tmx- and EE_2+Tmx-treated fish (Fig. 4F). After the recovery period, the expression levels of *dmrt1* gene were down-regulated in the fish that had been exposed to EE_2 or EE_2+Tmx (Fig. 4F).

The expression levels of *fshr* (Fig. 5A), *lhr* (Fig. 5B) and *gper* (Fig. 5D) genes decreased after 5 days of Tmx or EE_2+Tmx dietary intake, while the expression of *era*

was not affected by any treatment (Fig. 5C). After 25 days of treatment, the expression levels of *lhr* were similar to control ones (Fig. 5B), while those of *fshr* were up-regulated by all the treatments (Fig. 5A).The expression levels of *era* (Fig. 5C) and *gper* (Fig. 5D) were increased by Tmx and EE_2+Tmx, the up-regulation seen in *gper* gene expression being more pronounced in the EE_2+Tmx-treated than in Tmx-treated fish (Fig. 5A-D). However, after the recovery period, the expression levels of *fshr* were up-regulated in the EE_2-treated group (Fig. 5A), and those of *lhr* were up-regulated in the EE_2-treated group and down-regulated in the EE_2+Tmx treated group (Fig. 5B), while expression levels of *era* and *gper* had returned to control levels.

EE_2 and Tmx differently affect the immune response in the gonad

Acidophilic granulocytes and B lymphocytes (IgM+ cells) were revealed by immunolabelling with G7 (Fig. 6) and anti-IgM (Fig. 7), respectively. In control fish, scattered or, occasionally, small groups of acidophilic granulocytes were located in the interstitial tissue of the testicular area (Fig. 6A) or in the connective tissue that limited the ovarian and testicular areas of the gonad. Although the localization of these cells did not change at any time during the experimental period in any treatment, they were more numerous after 25 days of EE_2, Tmx and EE_2+Tmx dietary intake (Fig. 6B,C). Interestingly, after the recovery period, this increase was not observed in fish fed with Tmx (Fig. 6E) but was evident in fish that had been fed with EE_2+Tmx and, especially, EE_2 (Fig. 6D,F). IgM+ cell (Fig. 7) recruitment was triggered only by EE_2 after 5 days of exposure (Fig. 7B). However, after the recovery period, an increase in the amount of IgM+ cells was observed in the fish that had been fed with diets containing EE_2 or EE_2+Tmx (Fig. 7C,D).

As regards the pro-inflammatory cytokine genes, *il1b* and *tnfa*, the former was up-regulated by EE_2 and Tmx after 25 days of exposure (Fig. 8A), while the *tnfa* was down-regulated by Tmx after 5 days (Fig. 8B). Both cytokine gene expression levels were down-regulated after 5 days and up-regulated after 25 days of EE_2+Tmx dietary intake. After the recovery period, the expression levels of *tnfa* had only increased in the EE_2 treated fish (Fig. 8B). Regarding the expression of the anti-inflammatory cytokine gene *tgfb1*, its transcription was inhibited after 5 days of exposure with all the treatments (Fig. 8C). However, the *tgfb1* expression was up-regulated after 25 days of Tmx and EE_2+Tmx dietary intake and after the recovery period in all treated groups.

When the expression pattern of two metalloproteinase genes, *mmp 9* (Fig. 8D) and *mmp13* (Fig. 8E), was analyzed, they were seen to have an almost inverted pattern

of expression. Thus, after 5 and 25 days of EE_2 exposure, the transcription of *mmp9* gene had increased, whilst the transcription of *mmp13* gene had decreased. Tmx dietary intake for 5 days triggered a decrease and an increase of the transcription levels of *mmp9* and *mmp13* genes, respectively, while after 25 days of treatment, only the transcription levels of *mmp9* gene increased. Interestingly, the expression of *mmp13* was down-regulated after 5 days of EE_2+Tmx dietary intake, while the expression of both *mmp9* and *mmp13* was up-regulated after 25 days of this treatment. On the other hand, after the recovery period, the expression levels of *mmp9* were higher in all the treated groups compared with the control, while *mmp13* transcription was only increased in the fish treated with EE_2 or EE_2+Tmx.

Regarding the expression of some antigen recognition genes, the expression levels of *mhc1a* (Fig. 8F) or *tlr9* (Fig. 8G) genes decreased after 5 days of EE_2, Tmx or EE_2+Tmx treatments and after 25 days of EE_2 dietary intake. Interestingly, the *mhc1a* transcription levels increased after 25 days of Tmx or EE_2+Tmx dietary intake. After the recovery period, both genes were up-regulated in the EE_2 treated group, while only the *mhc1a* gene was up-regulated in the Tmx and EE_2+Tmx treated groups.

Discussion

The expression level of the hepatic *vtg* has been widely used as a marker of estrogenic endocrine disruption in fish (Hiramatsu el al., 2005; Bugel et al., 2013; Genovese et al., 2014; Hultman et al., 2015; Saunders et al., 2015). The present study shows that both EE_2, as described previously (Cabas et al., 2011, 2013), and Tmx act as estrogenic endocrine disruptors in gilthead seabream males, since they up-regulate the expression of the hepatic *vtg* gene. Tmx also increased the *vtg* gene expression in some fish species (Sun et al., 2007; Benninghoff and Williams, 2008) and the VTG concentration in fish homogenates of zebrafish males (Baumann et al., 2014), although it did not have any effect in other fish species (Leaños-Castañeda et al., 2002; Maradonna et al., 2009). However, when Tmx was applied with EE_2, a reduction in the EE_2-induced *vtg* gene expression was observed, as reported in other fish species upon exposure to Tmx and other estrogenic compounds (Leaños-Castañeda et al., 2002; Benninghoff and Williams, 2008; Maradonna et al., 2009; Sun et al., 2011b). Our results indicate that Tmx activates some ERs at the same time as it reduces their availability for binding to EE_2, which is consistent with the formation of the relatively stable Tmx-ER complex described by Jordan et al. (1977).

Although exposure to EE_2 does not affect plasmatic levels of T and E_2 in some fish (Swapna and Senthilkimaran, 2009; Colli-Dula et al., 2014), in gilthead seabream adult males serum levels of the main fish sex steroid vary according to the reproductive stage of the specimens and the EE_2 concentrations and time point analyzed (Cabas et al., 2011, 2013). In this sense, no effect of EE_2 was observed in our study during treatment, although it promoted an increase in T levels after the recovery period. As regards Tmx, an increase in androgen plasma levels was observed in our study as also occurs in carp males (Bottero et al., 2005), while in addition to this effect, we observed an increase in E_2 serum levels upon short term treatment. However, longer treatment led to the restoration of control levels of the sex steroid, which indicates that fish adjusted to sustained exposure. Interestingly, combined exposure to EE_2 and Tmx counteract their individual effects on serum androgen levels, which suggests an antagonistic action of EE_2 and Tmx beyond their direct competition to bind some ERs.

Despite the unaffected steroid plasma levels seen in EE_2-treated fish, our data on steroidogenic enzyme gene expression levels reflect an alteration in the steroidogenesis capability of the specimens, as occurs in other fish species upon EE_2 exposure (Sridevi et al., 2013; Colli-Dula et al., 2014). Thus, the gene expression of most of the steroidogenic enzymes analyzed in this study fell after short exposure to EE_2 and Tmx, whether separately or in combination. However, after longer treatment, the effects of Tmx became the opposite of those promoted by EE_2 but were reinforced when both compounds were applied together. Only the *cyp19a1a* gene showed a different expression pattern, being down-regulated by Tmx, as reported in other fish (Kitano et al., 2007) and up-regulated by EE_2+Tmx. In medaka males, the transcription of *star*, *cyp11a* and *cyp19a* genes was up-regulated by EE_2+Tmx, but not by EE_2 exposure, which led to the suggestion of a compensatory feedback in response to the anti-estrogenic property of Tmx (Sun et al., 2011b). Our results, however, underline the complexity of the action mechanisms of Tmx, which may, as occurs in mammals, bind to more than one ER (Fitts et al., 2011) and recruit tissue-specific co-regulators that would determine different downstream effects, depending on the cellular context (McDonnell and Wardell, 2010).

The depletion of *dmrt1* gene expression in mammals leads to the loss of mitotic germ cells, which precociously enter meiosis (Don et al., 2011). In addition, we found that EE_2 decreases the expression levels of *dmrt1* gene, concomitant with a depletion of meiotic cells in testis (Cabas et al., 2011, 2013). Thus, the consumption of meiotic cells

observed in the testis of fish exposed to EE_2, as reported also in catfish upon EE_2 exposure (Swapna and Senthilkimaran, 2009), or EE_2+Tmx for 25 days was probably triggered by the initial decrease in the expression levels of *dmrt1*. In addition, E_2 caused the mitotic division of germ cells in testicular fragments of Japanese eel *in vitro* (Miura et al., 1999). Hence, initial decreases in the expression levels of *dmrt1* in fish exposed to Tmx did not lead to the subsequent exhaustion of meiotic cells, probably due to the high serum levels of E_2 occurring in these fish. Accordingly, the renewal of mitotic cells after the recovery period in gilthead seabream treated with EE_2+Tmx could have been triggered by the increase in the expression levels of *dmrt1*, aided by the high expression levels of *era* and *gper*, which would imply a certain estrogenic effect even though E_2 in serum is at control levels, by the end of treatment period. All these data support that E_2 has a role in spermatogonia stem cell renewal in gilthead seabream as also suggested in Japanese eel (Miura et al., 1999). Moreover, if 11KT is needed for meiosis to begin, as established for Japanese eel (Miura et al., 1999), the high 11KT serum levels in Tmx-treated fish could have prevented the accumulation of degenerative cysts in the rest of the treated groups following short treatment, which would be explained by impaired spermatogonia stem cell divisions promoted by the decrease in expression levels of *dmrt1* and the initiation of meiosis. Moreover, our data demonstrate that Tmx partially neutralizes the effects of EE_2 on spermatogenesis, accelerating the recovery of the spermatogenic process upon the cessation of exposure, although it was not able to prevent the increase in interstitial tissue caused by EE_2 treatment reported in fish (Elias et al., 2007; Kaptaner and Ünal, 2010) and also observed in our study.

Although no differences between control and Tmx-treated fish were observed in testis morphology after 25 days of treatment, the spermatogenic process was somehow affected by Tmx, as shown by the increased sperm concentration and higher motility index. In addition, in Tmx-treated fish the expression levels of *cyp19a1a* gene decreased, while those of *dmrt1* gene increased, which have been described as being necessary to induce testicular differentiation and for the maintenance of testicular function in fish, respectively (Marchand et al., 2000; Liarte et al., 2007; Guiguen et al., 2010). The same was recorded during female-to-male sex reversal caused by Tmx in the Southern catfish (Liu et al., 2010). FSH regulates Sertoli cell proliferation in sea bass (Mazón et al., 2014) and the expression of *fshr* gene has been related with this process and with early spermatogenesis (Rahman et al., 2003; Rocha et al., 2009). In gilthead seabream, the sharp increase in *fshr* expression levels after 25 days of EE_2+Tmx treatment could have helped the restoration of mitotic activity in the testis of these fish

after the recovery period. Thus, our data point to the existence of a testicular network that involves the *dmrt1, era, gper* and *fshr* genes, whose rates of expression could determine the rates of mitotic proliferation and entrance of germ cells into spermatogenesis, aided by relevant plasma levels of E_2 and 11KT.

EE_2 affects the recruitment of leukocytes and the regulation of the cytokine network in fish gonads (Cabas et al., 2011, 2013; Seemann et al., 2013), up-regulating the expression of genes coding for the molecules involved in gilthead seabream (Cabas et al., 2011, 2013). In the present study, the expression patterns of pro- and anti-inflammatory cytokines (*il1b, tnfα* and *tgfb*) were stimulated or decreased according to the time of exposure, as occurred in fish treated with Tmx. However, in all cases, treatment with EE_2+Tmx enhanced the effect of Tmx alone, indicating a non-competitive but synergic action of both compounds on the expression of these genes. As regards the expression patterns of *mmp* genes, those of *mmp9* agree with the recruitment of acidophilic granulocytes in the testicular tissue of fish treated with EE_2, Tmx and EE_2+Tmx. On the other hand, it has been suggested that EE_2 stimulates the ability of the gonad to recognize and respond to pathogens (Cabas et al., 2011). In the present study, however, the expression of *tlr9* and *mch1a* genes was up-regulated by EE_2 only when applied together with Tmx for 25 days or after the recovery period. These data suggest that the effect of endocrine disrupter on this process might not only depend on the compound itself but, on the physiological state of the individuals.

The long-term effects of Tmx, supplied alone or with EE_2, on most parameters studied were contrary to those caused by short treatment. Changes in the effects of Tmx over time have also been described during tumour treatment (McDonnell and Wardell, 2010). Interaction with different ERs at different times could be involved in these striking responses, as various *er* genes are expressed in fish, including those coding for nuclear ERα, ERβ-I and ERβ-II (Nelson and Habibi, 2013) and the membrane-associated GPER (Liu et al., 2009; Pang and Thomas, 2010). However, the similar response of genes coding for ERα and GPER to the treatments could indicate a homeostatic response to sustained exposure, resulting in an up-regulation of the expression of the genes involved. Also, a non-receptor mediatory mechanism, such as an inhibitory effect of the chemicals on the activity of steroidogenic enzymes, as suggested by Colli-Dula et al. (2014), could contribute to the imbalance in the reproductive process.

In the present study, the effects promoted by Tmx and/or EE_2 were neutralized after the recovery period in the case of *era* and *gper* genes. However, neither the expression levels of *vtg* gene, the genes coding for the steroidogenic enzymes analyzed, nor serum steroid levels were restored to control values after 25 days of recovery. Studies on the ability to recover from EE_2 exposure, mostly carried out on zebrafish, have focused on the sexual differentiation and reproductive capacity (Hill and Janz, 2003; Nash et al., 2004; Schafers et al., 2007; Baumann et al., 2014). Developing catfish maintained high expression levels of genes coding for steroidogenic enzymes up to 300 days after a 50-day exposure to EE_2 (Sridevi et al., 2013). Further studies are needed to clarify the action mechanisms of these disruptive compounds and the ability of fish, particularly mature fish, to recover from their effects.

In conclusion, our data indicate that Tmx acts as an endocrine disruptor in gilthead seabream males. It has estrogenic effects, such as the up-regulation of expression levels of hepatic *vtg* and gonadal *era and gper* genes and the increase of E_2 serum levels. In addition, Tmx has some effects that do not fit with an estrogenic action, such as an increase in serum levels of androgens, and the up- and down- regulation of expression levels of *dmrt1* and *cyp19a1a* genes, respectively. However, when combined with the estrogenic compound EE_2, Tmx may counteract (*vtg, dmrt1*) or enhance (*fsh, era, gper*) its effects on gene expression levels. It was also found that the disruptive effect of EE_2 and/or Tmx on the reproductive process is not reversible after a 25 day recovery period, since expression levels of hepatic *vtg* and other parameters studied were still disturbed after this time.

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Author contributions

Conceived and designed the experiments: MPGH, AlGA, ECP, AGA. Performed the experiments: MPGH, MCR, IC, ECP. Analyzed the data: MPGH, MCR, IC, AlGA, ECP, AGA. Contributed reagents/materials/analysis tools: AlGA, ECP, AGA. Contributed to the discussion of the result and the writing of the manuscript: MPGH, AlGA, ECP, AGA.

Author competing interests

The author(s) declare that they have no competing interests.

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Figure legends

Figure 1: *EE_2, Tmx and EE_2+Tmx increase the transcription levels of vtg gene and differently affect the serum levels of main steroid hormones in gilthead seabream males.* The transcription levels of *vtg* in the liver (A) and the serum levels of T (B), 11KT (C) and E_2 (D) of gilthead seabream males treated with 0 (control), 5 µg EE_2/g food, 100 µg Tmx/g food or 5 µg EE_2+100 µg Tmx/g food for 5 and 25 days and after 25 days of

reverting the commercial diet (d25 recovery). The asterisks denote statistically significant differences between the groups according to Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2: *EE_2, Tmx and EE_2+Tmx modulate the expression of genes coding for steroidogenic-relevant molecules in the gonad of gilthead seabream males*. The specimens were treated with 0 (control), 5 µg EE_2/g food, 100 µg Tmx/g food or 5 µg EE_2+100 µg Tmx /g food for 5 and 25 days, and after 25 days of reverting to the commercial diet (d25 recovery). The mRNA levels of *star* (A), *cyp11a1* (B), *cyp11b1* (C), *hsd11b* (D), *cyp19a1a* (E), *srd5a* (F), and *hsd3b* (G) were determined in the gonad by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent means ± S.E.M. of the gene expression from 5 independent fish/group and time. The asterisks denote statistically significant differences between the groups according to Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3: *Effects of EE_2, Tmx and EE_2+Tmx dietary intake on the testicular morphology of gilthead seabream males.* Paraplast embedded sections (A-E) of the gonad of gilthead seabream males treated with 0 (control, C) (A) or 5 µg EE_2/g food (B, D), or 5 µg EE_2 + 100 µg Tmx /g food (C, E) for 5 (A, B) and 25 (C) days and after 25 days of reverting to the commercial diet (rp, D, E) stained with hematoxylin-eosin (HE). White arrowheads: primary spermatogonia; white arrows: degenerative cysts; asterisk: spermatogonia cysts; Sc: spermatocytes, Sz: spermatozoa. Scale bar = 35 µm.

Figure 4: *Effect of EE_2, Tmx and EE_2+Tmx on cell testicular proliferation and on the dmrt1 gene expression.* Paraplast embedded sections immunostained with anti-proliferating cell nuclear antigen (anti-PCNA) serum (A-E) and the transcription levels of *dmrt1* gene (F) of the gonad of gilthead seabream males treated with 0 (control, C) (A), 5 µg EE_2/g food (C, D), 100 µg Tmx/g food (B) or 5 µg EE_2+100 µg Tmx /g food (E) for 5 (A, B) and 25 (C) days and after 25 days of reverting to commercial diet (rp, D, E). (A-E) white arrowheads: spermatogonia stem cells; white arrows: proliferative primary spermatogonia; asterisks: cyst of proliferative spermatogonia: black arrows: proliferative Sertoli cells. Scale bar = 35 µm. The mRNA levels of *dmrt1* (F) were determined in the gonad by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent means ± S.E.M. of the gene expression from 5 independent fish/group and time. The asterisks denote statistically significant differences between the groups according to Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5: *EE_2, Tmx and EE_2+Tmx modulate the expression of genes coding for hormone receptors in the gonad of gilthead seabream males.* The specimens were

treated with 0 (control), 5 µg EE_2/g food, 100 µg Tmx/g food or 5 µg EE_2+100 µg Tmx/g food for 5 and 25 days and after 25 days of reverting to the commercial diet (d25 recovery). The mRNA levels of *fshr* (A), *lhr* (B), *era* (C) and *gper* (D) were determined in the gonad by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent means ± S.E.M. of the gene expression from 5 independent fish/group and time. The asterisks denote statistically significant differences between the groups according to Tukey's test. ND, not detected; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 6: *EE_2, Tmx and EE_2+Tmx recruited acidophilic granulocytes into the testis of gilthead seabream.* Paraplast embedded sections of the testis of gilthead seabream males treated with 0 (control, C) (A), 5 µg EE_2/g food (D), 100 µg Tmx/g food (B, E) or 5 µg EE_2+100 µg Tmx/g food (C, F) for 5 (A) and 25 (B, C) days and after 25 days of reverting to the commercial diet (rp, D-F) immunostained with the serum against gilthead seabream acidophilic granulocytes (G7, black arrows). Scale bar = 35 µm.

Figure 7: *EE_2, and EE_2+Tmx, but not Tmx, recruited B-lymphocytes into the testis of gilthead seabream.* Paraplast embedded sections of the testis of gilthead seabream males treated with 0 (control, C) (A), 5 µg EE_2/g food (B, C) or 5 µg EE_2 + 100 µg Tmx /g food (D) for 5 days (A, B) and after 25 days of reverting to the commercial diet (rp, C, D) immunostained with the serum against gilthead seabream IgM. Black arrows: B-lymphocytes stained with the anti-gilthead seabream IgM serum. Scale bar = 35 µm.

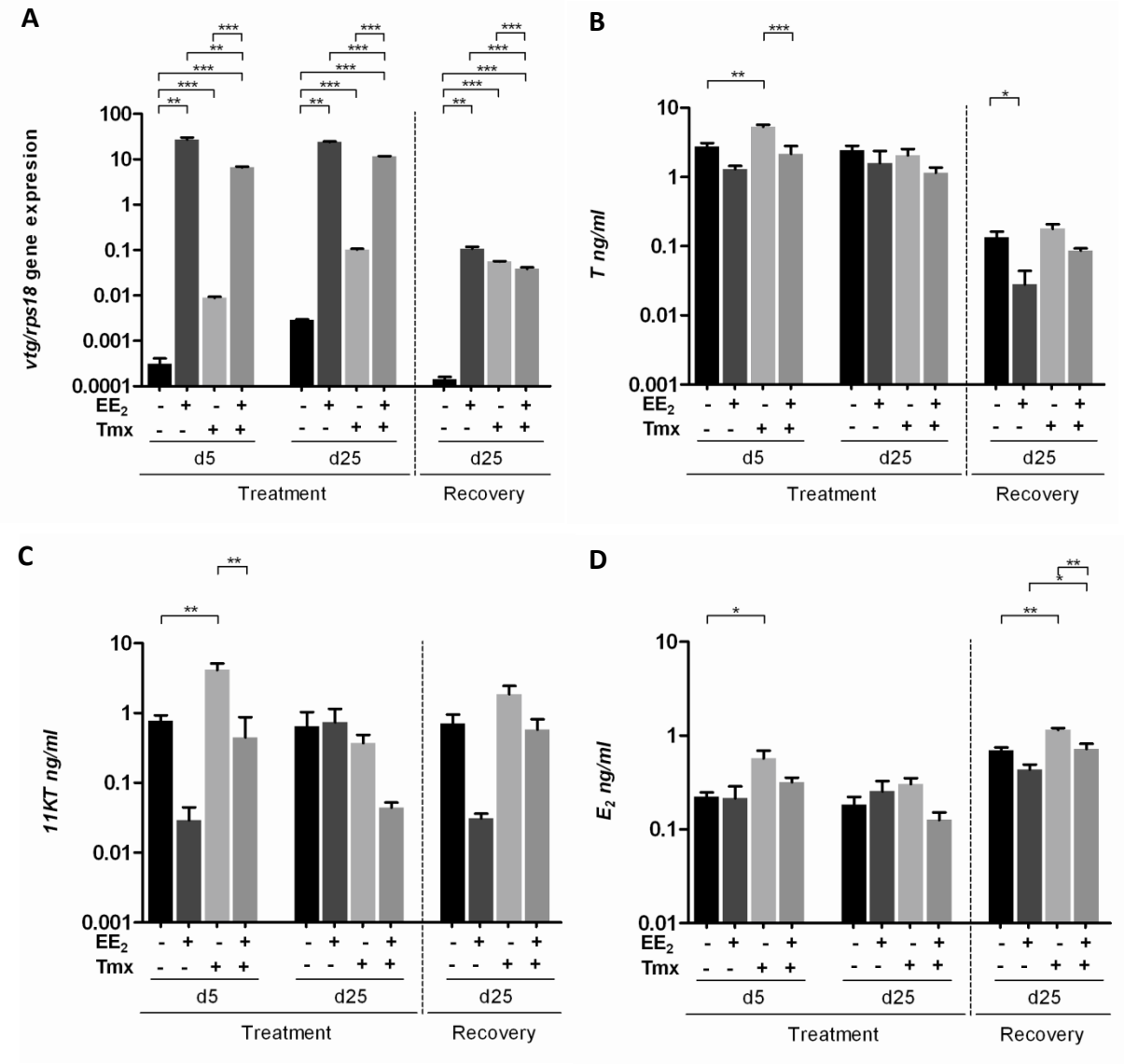
Figure 8: *EE_2, Tmx and EE_2+Tmx modulate the expression of genes coding for some immune-relevant molecules in the gonad of gilthead seabream males*. The specimens were treated with 0 (control), 5 µg EE_2/g food, 100 µg Tmx/g food or 5 µg EE_2+100 µg Tmx/g food for 5 and 25 days and after 25 days of reverting to the commercial diet (d25 recovery). The mRNA levels of *il1b* (A), *tnfa* (B), *tgfb1* (C), *mmp9* (D), *mmp13* (E), *mhc1a* (F), and *tlr9* (G) were determined in the gonad by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent means ± S.E.M. of the gene expression from 5 independent fish/group and time. The asterisks denote statistically significant differences between the groups according to Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

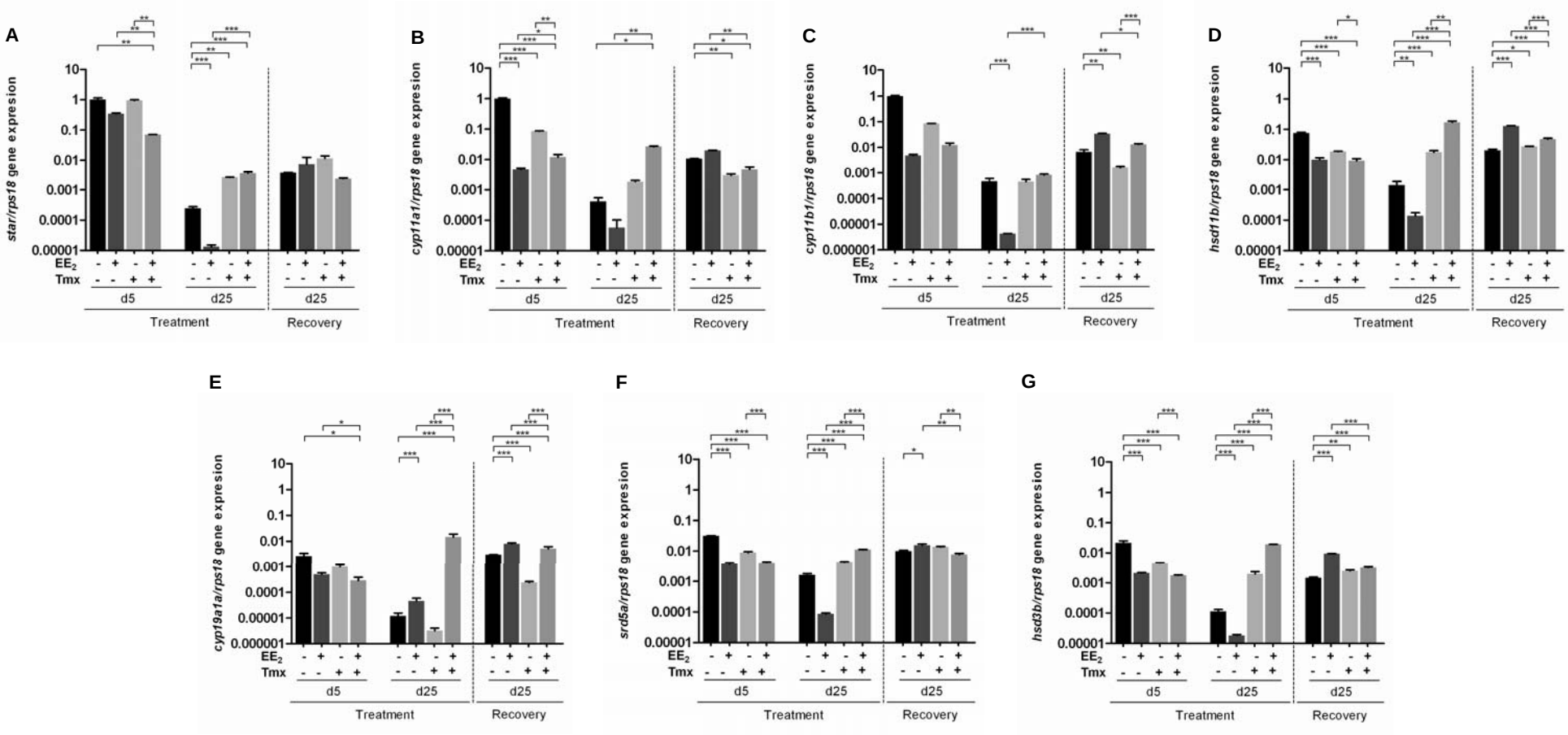
Table 1: Gene accession numbers and primer sequences used for gene expression analysis by real time PCR.

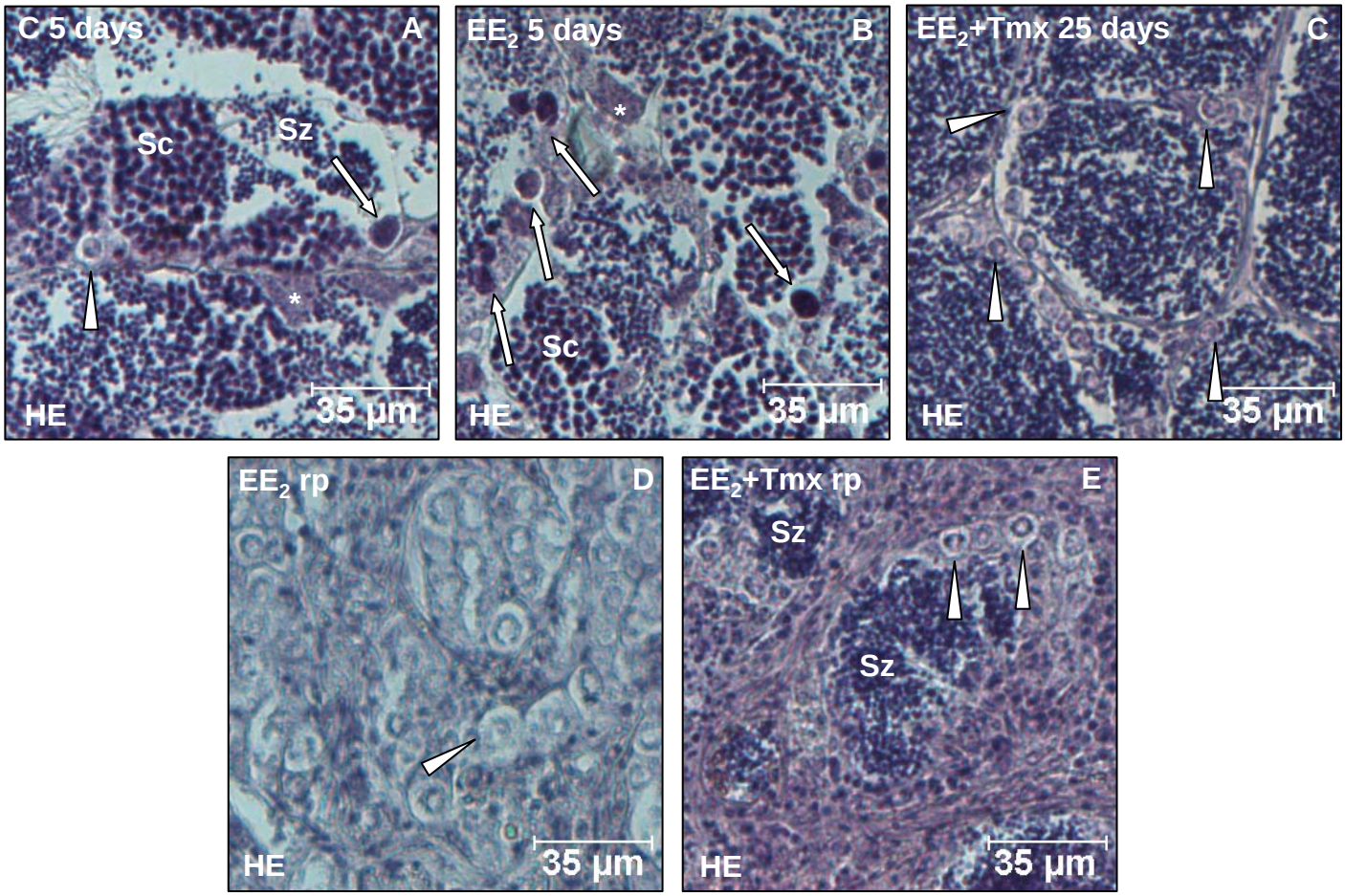
Gene	Accession number	Name	Sequence (5`-3')
vtg	AF210428	F1	CTGCTGAAGAGGGACCAGAC
		R1	TTGCCTGCAGGATGATGATA
star	AM905934	F1	ACATCGGGAAGGTGTTCAAG
		R1	TCTCTGCAGACACCTCATGG
cyp11a1	FM159974.1	F	CGCTGCTGTGGACATTGTAT
		R	CATCATGTCTCCCTGGCTTT
cyp11b1	FP332145	F	GCTATCTTTGGACCCCATCA
		R	CTTGACTGTGCCTTTCAGCA
hsd11b	AM973598	F	AGACATGGGCAACGAGTCAG
		R	TCCACATCTCCCTCCCACAT
cyp19a1a	AF399824	F2	CAATGGAGAGGAAACCCTCA
		R2	ATGCAGCTGAGTCCCTGTCT
srd5a	AM958800	F	TGCACTTTCGTGACTCTGCT
		R	TTTCGCACAAGACGTCCAGA
hsd3b	HS985587	F	GGAGGACAAACTGGTGGAGG
		R	ACATTCTCCGTTCCGGTGAC
dmrt1	AM493678	F	GATGGACAATCCCTGACACC
		R	GGGTAGCGTGAAGGTTGGTA
fshr	AY587262	F2	TCCCACTACGGATCCTCATC
		R2	AACGGGAACAGTCAGTTTG
lhr	AY587261	F2	ATACACGACCACGCATTCAA
		R2	CGCCGGTAACTTCTTGAGAG
era	AF136979	F	GCTTGCCGTCTTAGGAAGTG
		R	TGCTGCTGATGTGTTTCCTC
gper	HG004163	F1	GGCTGCCAGAGAATGTCTTC
		R1	GTGGCCTGTGAGTGGGTAGT
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
tnfa	AJ413189	FE1	TCGTTCAGAGTCTCCTGCAG
		RE3	CATGGACTCTGAGTAGCGCGA
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA
		R	GCCTGAGGAGACTCTGTTGG
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
mhc1a	AY292461	F	CCAGAGCTTCCCTCAGTGTC
		R	CATCTGGAAGGTTCCATCGT
tlr9	AY751798	F2	GGAGGAGAGGGACTGGAT
		R2	GATCACACCGTCACTGTCTC
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC

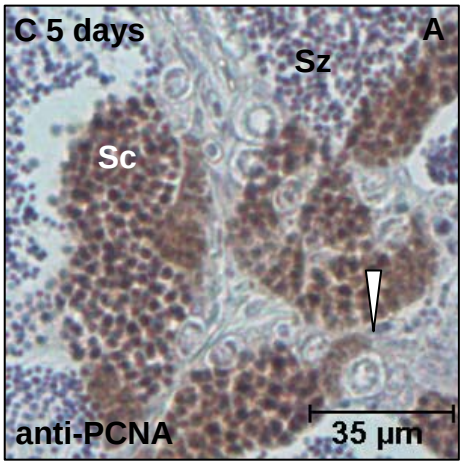
Table 2. Effects of the dietary intake of 5 µg 17α-ethynylestradiol (EE_2)/g food, 100 µg tamoxifen (Tmx)/g food or 5 µg EE_2 +100 µg Tmx/g food during 5 and 25 days (Treatment) and after resuming normal diet (Recovery) during 25 days on volume of seminal fluid (mL), sperm concentration (cell/mL) and motility index. Data represent means ± SEM of six independent fish per group. Asterisks denote statistically significant differences between treatment and control groups according to a Student t test (*P ≤ 0.1; **P≤0.05). ND: not detected

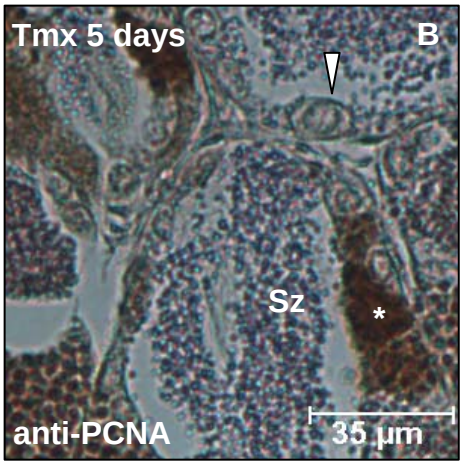
	Volume of seminal fluid (mL)			Sperm concentration (cell/mL)			Sperm motility index		
	Treatment		Recovery	Treatment		Recovery	Treatment		Recovery
Treatment	5 days	25 days	25 days	5 days	25 days	25 days	5 days	25 days	25 days
Control	0.83±0.14	1.03±0.31	0.9±0.24	16.37±3.0	(9.32±0.60)x10^3	(7.66±0.44) x10^3	0.83±0.31	0.33±0.25	2.08±0.27
EE_2	0.02±0.02**	0.68±0.45	ND	ND	(4.54±2.96) x10^3**	ND	ND	0.67±.033	ND
Tmx	0.73±0.18	2.67±1.19	2.65±0.88*	9.17±2.29	(13.33±0.75) x10^3**	(9.81±0.60) x10^3**	1.38±0.33	1.42±0.20*	1.58±0.30
EE_2+Tmx	0.73±0.23	0.08±0.07**	ND	8.5±1.95**	(3.31±2.88) x10^3**	ND	1.0±0.37	0.58±0.37	ND

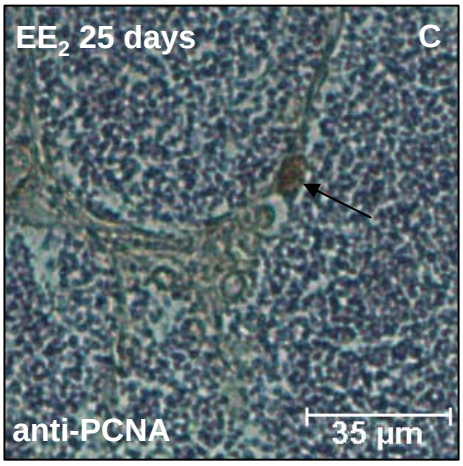

A
vtg/rps18 gene expresion
100
10
1
0.1
0.01
0.001
0.0001
EE2
Tmx
d5
d25
d25
Treatment
Recovery
B
T ng/ml
10
1
0.1
0.01
0.001
C
11KT ng/ml
D
E2 ng/ml

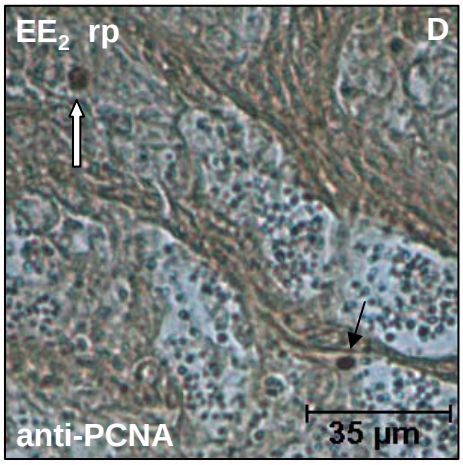

A
B
C
D
E
F
G
star/rps18 gene expresion
cyp11a1/rps18 gene expresion
cyp11b1/rps18 gene expresion
hsd11b/rps18 gene expresion
cyp19a1a/rps18 gene expresion
srd5a/rps18 gene expresion
hsd3b/rps18 gene expresion
EE2
Tmx
d5
d25
Treatment
Recovery

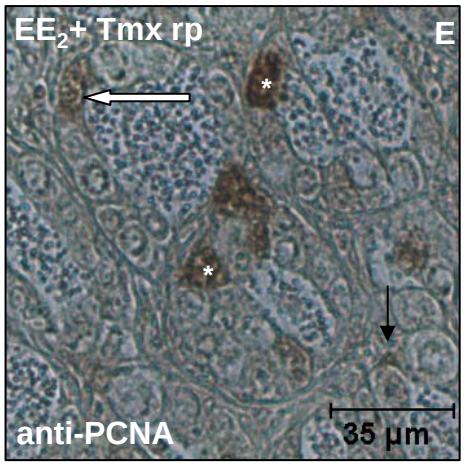

C 5 days
A
Sc
Sz
*
HE
35 µm
EE2 5 days
B
*
Sc
HE
35 µm
EE2+Tmx 25 days
C
HE
35 µm
EE2 rp
D
HE
35 µm
EE2+Tmx rp
E
Sz
Sz
HE
35 µm

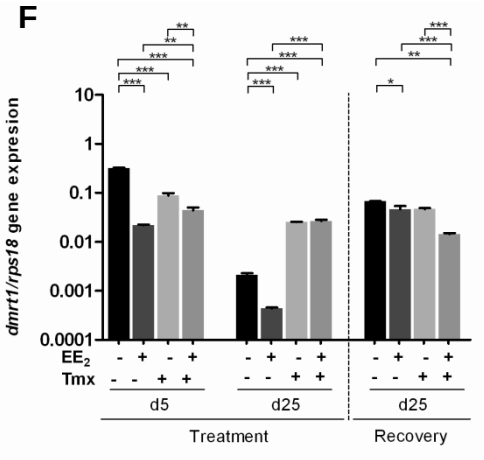

C 5 days
A
Sz
Sc
anti-PCNA
35 µm

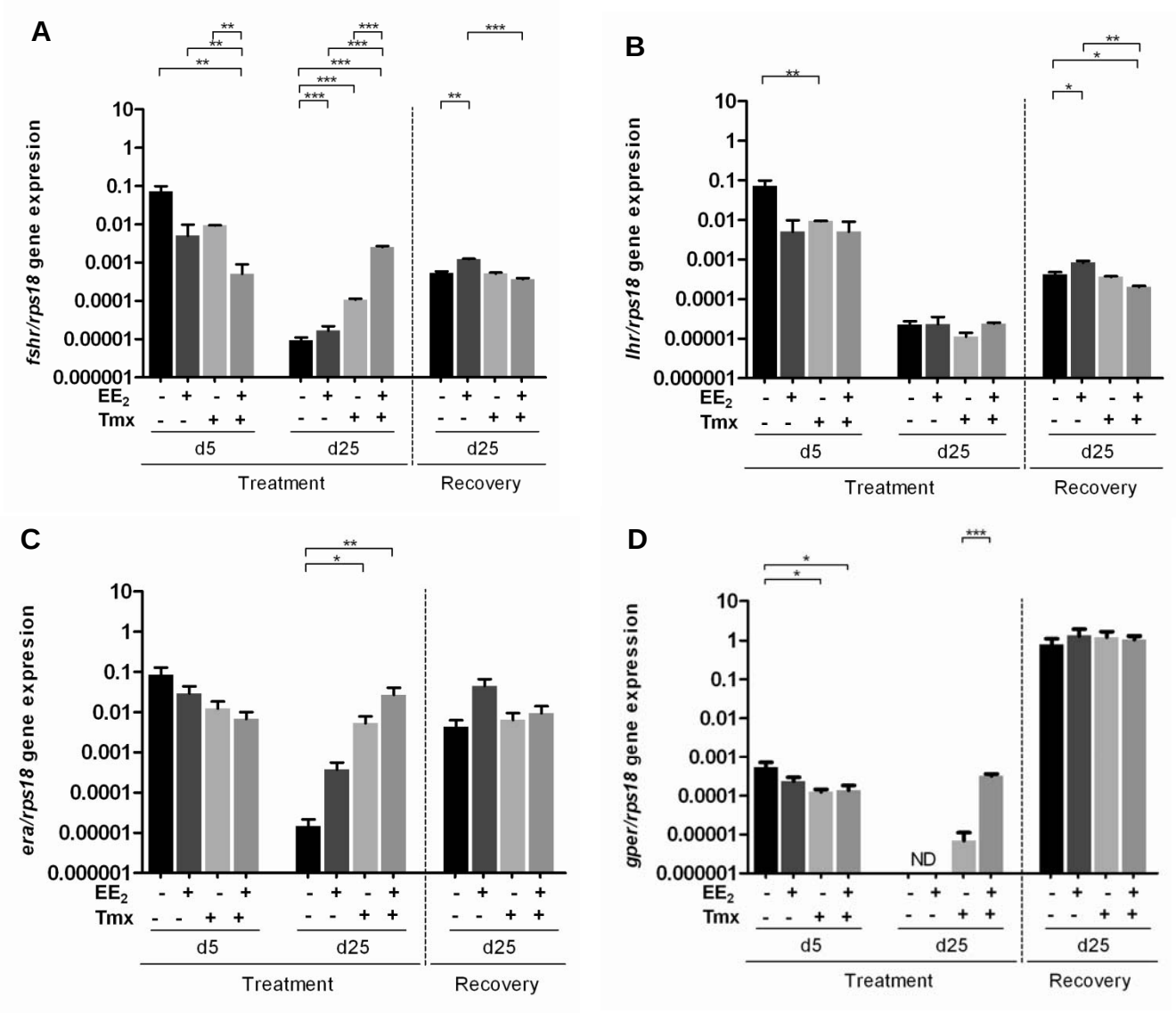

Tmx 5 days
B
Sz
*
anti-PCNA
35 µm

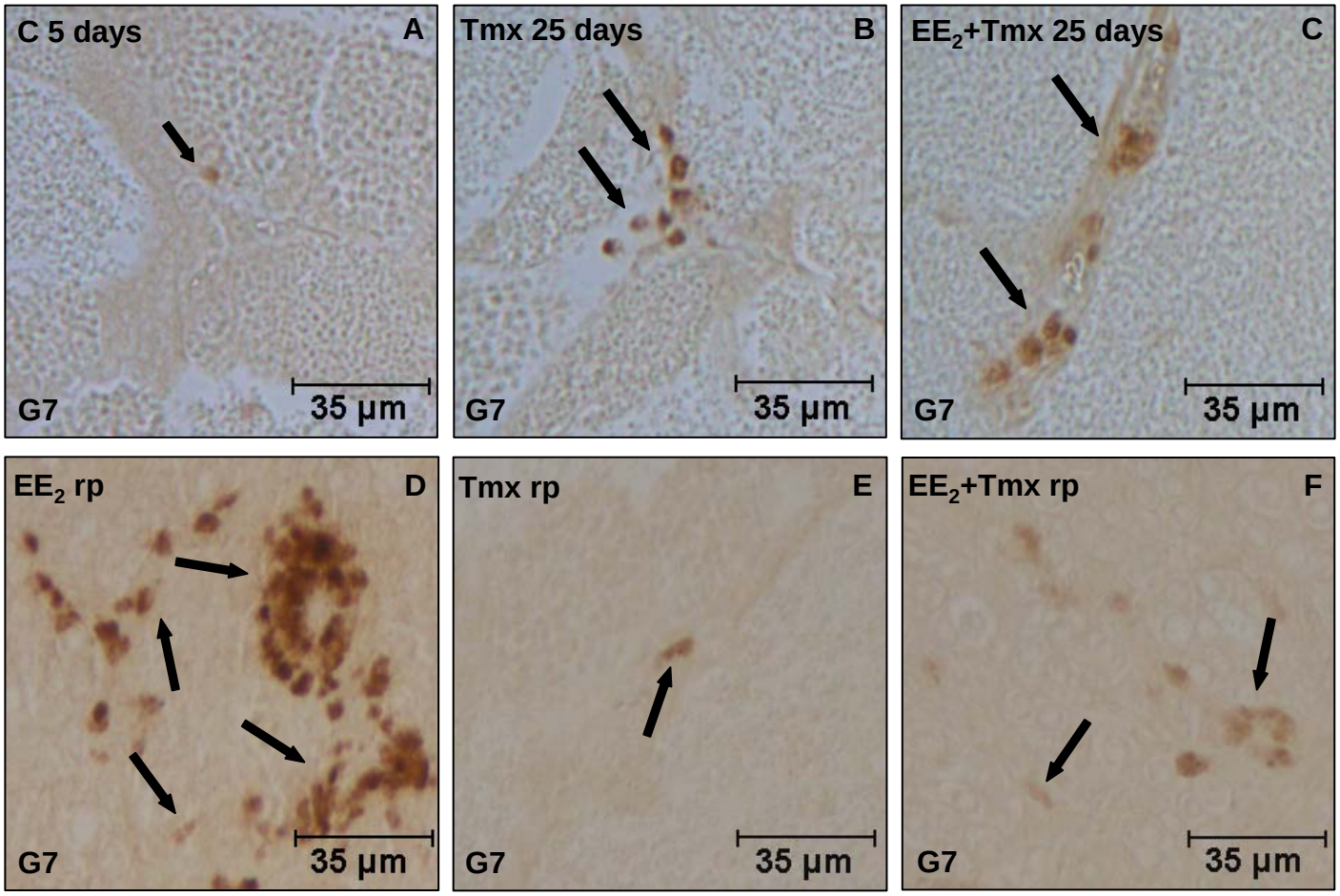

EE2 25 days
C
anti-PCNA
35 µm


EE2 rp
D
anti-PCNA
35 µm


EE2+ Tmx rp
E
*
*
anti-PCNA
35 µm


F
dmrt1/rps18 gene expresion
10
1
0.1
0.01
0.001
0.0001
EE2
Tmx
d5
d25
d25
Treatment
Recovery


A
fshr/rps18 gene expresion
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
EE2 - + - + - + - + - + - + - + - +
Tmx - - + + - - + + - - + + - - + +
d5
d25
d25
Treatment
Recovery
B
lhr/rps18 gene expresion
C
era/rps18 gene expresion
D
gper/rps18 gene expression
ND


C 5 days
A
G7
35 µm
Tmx 25 days
B
G7
35 µm
EE2+Tmx 25 days
C
G7
35 µm
EE2 rp
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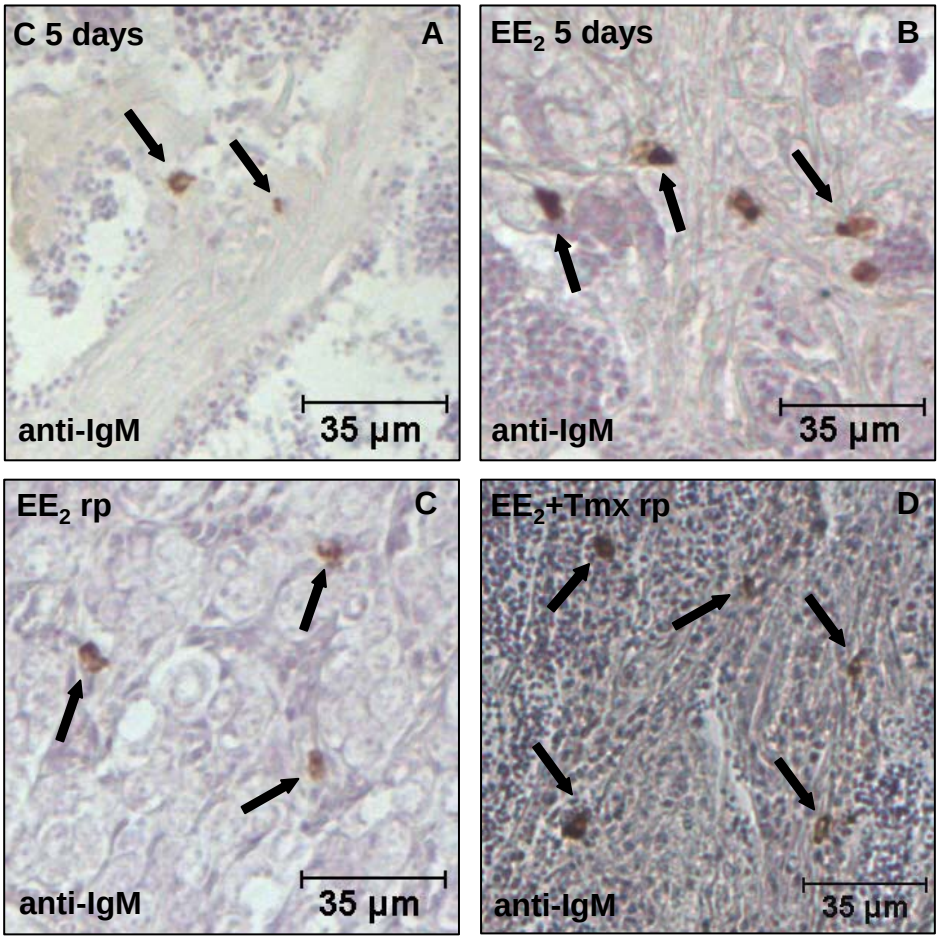
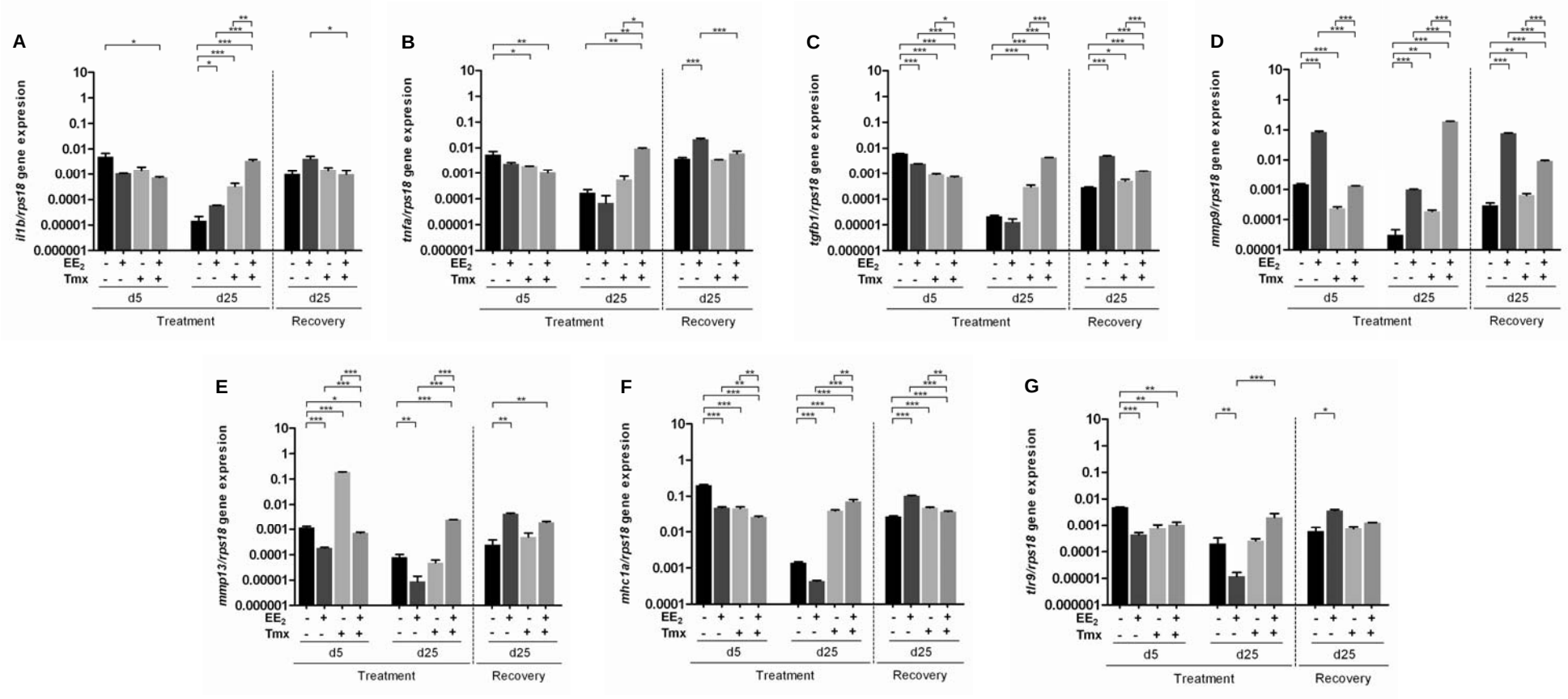
G7
35 µm
EE2+Tmx rp
F
G7
35 µm


C 5 days
A
anti-IgM
35 µm
EE2 5 days
B
anti-IgM
35 µm
EE2 rp
C
anti-IgM
35 µm
EE2+Tmx rp
D
anti-IgM
35 µm


A
il1b/rps18 gene expresion
B
tnfa/rps18 gene expresion
C
tgfb1/rps18 gene expresion
D
mmp9/rps18 gene expresion
E
mmp13/rps18 gene expresion
F
mhc1a/rps18 gene expresion
G
tlr9/rps18 gene expresion
EE2
Tmx
d5
d25
d25
Treatment
Recovery

Highlights

Tmx disrupts the gilthead seabream reproductive process including the gonad immune response

Tmx alters the expression profile of hepatic vitellogenin gene in males of a protandrous fish

Tmx can counteract or enhance the effects of EE_2 on reproductive several parameters

The disruptive effects of Tmx and/or EE_2 on reproduction are not reversed after a 25-day recovery period